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Detection by PCR of Human Papillomavirus Genotypes in Cervical Lesions of Senegalese Women

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In order to analyse human papillomavirus (HPV) infection in the Senegalese population, HPV DNA was sought in 65 women with evidence of cervical cytological abnormality and in 72 pregnant women. Ninety-four percent of the patients were positive for HPV DNA as compared to 24% of pregnant women. HPV 16 was detected in cervical smears in 42% of cases, HPV 18 in 39%, HPV 6 in 26%, HPV 11 in 15%, HPV 45 in 10%, HPV 52 in 3%, and HPV 31, HPV 33 and HPV 68 in 1.5%. HPV 16 and HPV 18 were detected in 16% and 7% respectively of pregnant women. HPV DNA of unknown type was detected in 6% of cases, and multiple HPV infections were observed in 28% of cases. Low risk genital HPVs (6/11) were detected in smaller proportions (17%) among high grade squamous intraepithelial lesions (SILs) than the low grade SILs (43%). High risk HPVs (16/18) were detected in high proportions both in low and high grade SIL lesions, though the highest frequency (70%) was observed among patients with high grade lesions. In conclusion, the results confirm that HPV infections are frequent in Senegal and that HPV 18 and 45 are detected in a high proportion of patients in Africa.

KEY WORDS: HPV typing, polymerase chain reaction, cervical cancer

INTRODUCTION

Twenty-eight distinct HPVs are associated with genital lesions. Some are associated strongly with the development of cervical cancer [Munoz and Bosch, 1992] and it is apparent that infections with types 16, 18, 31, 33 and 45 cause molecular pathogenesis leading to cancer of the cervix [Zur Hausen, 1994]. This is demonstrated by the facts that viral DNA is found in more than 90% of the tumours, tumour cells contain integrated viral DNA and such tumours reveal specific transcripts from viral open reading frames E6 and E7 which have been shown to induce cell proliferation and malignant transformation.

HPV genotypes have been grouped as high risk (HPV 16, 18 and 45), intermediate risk (HPV 31, 33) and low risk (HPV 6, 11) viruses for the development of cervical cancer. HPV 6 and HPV 11 are generally associated with condylomata accuminata and dysplasia that progress infrequently to cancer [Gissmann et al., 1983]. HPV 16 and 18 are both associated with cervical intraepithelial neoplasia (CIN) and with cervical carcinomas [Lorincz et al., 1986].

Cancer of the uterine cervix is recognized as the most common malignant tumour among African women [Parkin et al., 1988] and has been reported to be very frequent in Senegal [Tuyns and Quenum, 1982; Touré et al., 1984]. However, little information is available from African countries on the frequency of HPV types found in carcinomas and in the general population. HPV 16 and 18 were detected using nucleic acid hybridization asays in 17% of carcinoma in situ (CIN) in Kenya [Kreiss et al., 1992] and in 50% of cancers in Uganda [Schmauz et al., 1989]. HPV 16 and 18 were detected using the polymerase chain reaction (PCR) method in 47–68% of cervical cancers in South Africa and in Tanzania [Williamson et al., 1994; Ter Meulen et al., 1992].

In the present study, Senegalese women were investigated for the presence of genital papillomavirus by PCR on cervical cells obtained by scraping.

MATERIALS AND METHODS

Clinical Specimens and Patients

Cervical specimens from 137 women aged 13 to 71 years (mean 30 years) living in the Dakar area in Senegal were evaluated for HPV DNA. This group included 72 pregnant women (mean age 28 years) from M. Senghor's maternity unit of Yoff (Senegal) and 65 women (mean age 32 years) with a history of cervical lesions from the

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TABLE I. Oligonucleotide Primers for PCR Amplification

	Nucleotide sequence	Gene	Position	Amplimer length (bp)
HPV 6-a[a]	CACCTAAAGGTCCTGTTTCG	L1	4671–4690	279
HPV 6-b[a]	CGGTTTGTGACACAGGTAGC		4931–4950	
HPV 11-a	CCTTCTGTTTTGGAGGACTG	L1	6944–6963	113
HPV 11-b	GTTTTCGTTTGGGGGCTGGTA		7037–7057	
HPV 16-a	GGCTTTGGTGCTATGGAC	L1	6248–6265	414
HPV 16-b	AGTAGATATGGCAGCACA		6672–6689	
HPV 18-a[b]	AAGGATGCTGCACCGGCTGA	L1	6903–6922	216
HPV 18-b[b]	CACGCACACGCTTGGCAGGT		7100–7119	
HPV 31-a	ACACCACACGGAAGTGTGTAC	L1	421–440	162
HPV 31-b	ACACAACGGTCTTTGACACG		279–299	
HPV 33-a	CACACTTGTAACACCACAG	E7	748–768	497
HPV 33-b	ATAGACGTTCTACACGGGT		1226–1245	
HPV 35-a	AACGAAGCCACTGTCTACCT	L1	5592–5611	1441
HPV 35-b	GCACGCCTGCCTAATCTAAA		7014–7034	
HPV 45-a	TTCATGGCACACAATATTA	L1	5527–5545	1630
HPV 45-b	CATATTATTTCTTACTACG		7135–7153	
HPV 52-a	GATGAGTATGTGTCTCGCAC	L1	5715–5734	1379
HPV 52-b	TAGTTTGGGCCTAGCCTGTA		7074–7094	
HPV 58-a	CTGTGCCTGTGTCTAAGGTT	L1	5686–5705	1412
HPV 58-b	TTGCGTTTGGTGGATGGTG		7105–7123	
HPV 68-a	CAAGCAGGACATTCCTAAGG	L1	1690–1809	1318
HPV 68-b	GCTTAGAGGTAGATGCAGTAG		3088–3108	
HPV X1-a[c]	CAGGATGGT/G/CGAT/CATGGT	L1	6362–6378	440
HPV X1-b[c]	CAT/AG/TT/GTA/GATCTGCG/AT/G		6786–6802	
HPV X2-a	GTTGAACCGAAACCGGTTAG	E6	45–64	523
HPV X2-b	GTATGTAAGGCGTTGGCGCA		508–528	

[a]Park et al., 1991.
[b]Van den Brule et al., 1989.
[c]Williamson et al., 1991.

Le Dantec Hospital of Dakar. Cytological diagnosis was not obtained in 46 pregnant women and the remaining 26 had negative Pap smears. Abnormalities were graded according to the Bethesda System as low grade squamous intraepithelial lesions (SIL) and high grade SIL. Microscopic examination of Pap smears revealed 42 (65%) patients with low grade SIL and 23 (35%) patients with high grade SIL.

Detection and Typing of HPV DNA

Specimens were collected for cytological examination and DNA analysis by Ayre spatula. After preparation of microscope slides, the spatula was dipped in sterile phosphate buffered saline. Samples were kept frozen at −20°C until tested. Cytological diagnosis and HPV typing were carried out independently. Collected cells were lysed in 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5% SDS buffer. Total cellular DNA was prepared by proteinase K (10 μg/ml) digestion and phenol-chloroform extraction. The PCR assay was carried out on purified DNA in 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 50 mM KCl and 0.5% gelatin containing 200 μM of each deoxyribonucleoside triphosphate, 0.25 μg of the oligonucleotide primers (Table I), and one unit of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT). The primers specific for HPV 11, 16, 31, 33, 35, 45, 52, 58, and 68 and the consensus primers X2 were designed by the OLIGOTEST computer program [Beroud et al., 1990] and were evaluated against the 60 HPV sequences described in the Genebank using the Mac Vector computer program. Primers for HPV 6, HPV 18, and the consensus primers (X1) were those described by Park et al. [1988], van den Brule et al. [1989] and Williamson et al. [1991] respectively. The samples were subjected to 30 cycles of amplification using a DNA thermal cycler. For each cycle, denaturation was performed at 94°C for 30 s, annealing of primers at Tm −5°C for 30 s, and elongation at 75°C for 1 min. The mixture was incubated after the last cycle at 75°C for 3 min. PCR amplification was performed at least twice in each case. A negative control using water in place of the template was included in each PCR run and standard precautions were used to prevent carryover of DNA [Ljunggren and Kidd, 1991]. After amplification, 10 μl aliquots of each sample were electrophoresed in 1.6% agarose gel. The DNA was visualised by ethidium bromide staining (Fig. 1). Amplified DNA were subsequently transferred onto a nylon membrane and hybridized with a digoxigenin labelled probe obtained by random priming incorporation of digoxigenin-dUTP. The nylon membranes were washed twice for 5 min at room temperature with at least 50 ml of 2 × SSC, 0.1% SDS (w/v) and twice for 15 min at 68°C with 0.1 × SSC, 0.1% SDS (w/v). After molecular hybridization of this probe with the amplified HPV-DNA target, the hybrids were detected using an anti-digoxigenin alkaline-phosphatase conjugate and subsequent enzyme-catalyzed colour reaction with 5 bromo 4 chloro 3 indolyl phosphate and nitroblue tetrazolium salt (DNA labelling and detection kit, Boehringer Mannheim, Mannheim, Germany) (Fig. 2). HPV 35, 52, 58, and 68 were only investigated

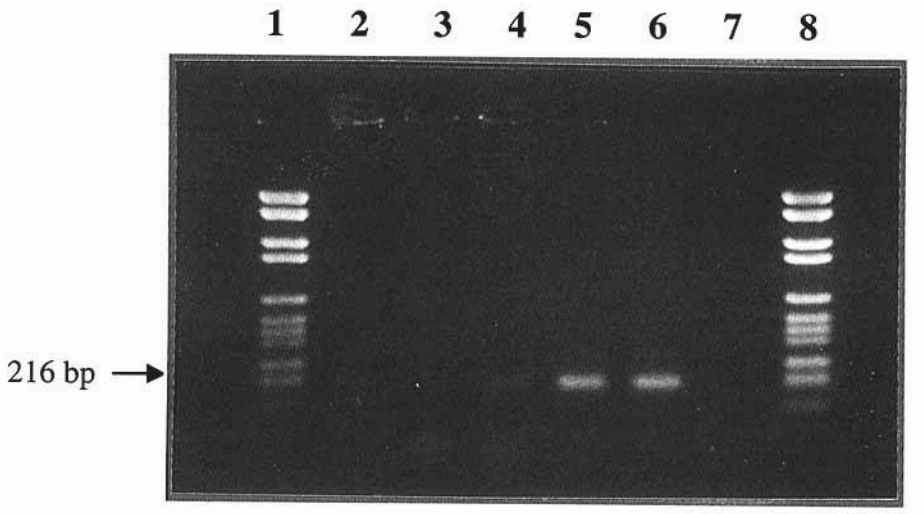

Fig. 1. Representative ethidium-bromide-stained agarose gel of amplified HPV DNA. **Lanes 1 and 8:** Molecular weight markers, **lanes 5 and 6:** HPV 18 DNA positive samples, **lane 7:** negative control.

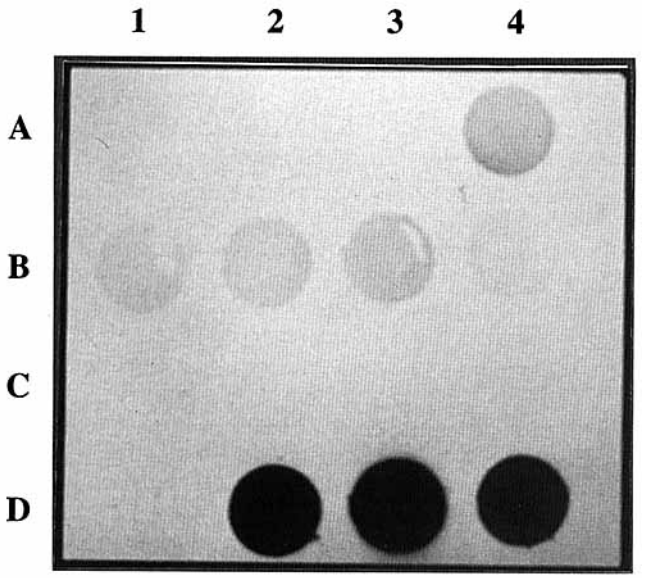

Fig. 2. Dot blot analysis of L1 amplified DNA using HPV 45 digoxigenin labelled probe. A2, A3, negative controls. D2, D3, positive controls. HPV 45 positive samples are on A4, B1, B2, B3 and D4.

in samples negative for HPV 6, 11, 16, 18, 31 and 33. To identify HPV 45, the specimens were PCR amplified using L1 consensus primers (X1) and the amplified DNA was then hybridized with a digoxigenin labelled HPV 45 probe obtained by random priming.

Statistical Analysis

Comparison of proportions were made using the χ^2 test with Yates' correction. Significance was set at 0.05.

RESULTS

HPV DNA was detected in 94% (61/65) of patients and in 23% (6/26) of pregnant women with cytologically negative Pap smears and in 24% (11/46) of pregnant women without a Pap smear (Table II). Among patients, HPV 16 was detected in 42% and HPV 18 in 39%. Only one patient had evidence of infection with HPV 31 and another of HPV 33. HPV 6 and 11 were detected respectively in 26% and 15% of patients. The 13 (19%) specimens which were amplified only with consensus primers were tested for HPV 45 by hybridization with the labelled HPV 45 probe. These 13 samples plus the 5 HPV negative for HPV 6, 11, 16, 18, 31 and 33 were then investigated for HPV 35, 52, 58 and 68 by PCR. Seven (10%) were found to be HPV 45 positive, two were HPV 52 positive (3%) and one was HPV 68 positive (1.5%). The remaining four specimens (6%) were categorized as unclassified HPVs.

It must be noted that a high level of multiple infections was observed: 12 double infections (6 HPV 16/18, 2 HPV 6/11, 1 HPV 16/33, 1 HPV 6/18, 1 HPV 11/16 and 1 HPV 11/18), four triple infections (2 were HPV 6/16/18, 1 was HPV 11/16/18 and 1 was HPV 16/18/31) and two quadruple infections (HPV 6/11/16/18). HPV 16 was detected in 40% and in 43%, and HPV 18 in 33% and 49% of patients with low and high grade SILs respectively. Simultaneous infection with HPV 16 and 18 was detected in eight (19%) patients with low grade lesions and in five (22%) with high grade SILs. Low risk HPVs (HPV 6/11) were detected in 43% of low grade SILs and in 17% of high grade SILs. HPV 6 and HPV 11 DNA were detected in 30% and 21% respectively of women with low grade SILs. The proportion decreased to 17% and 5% in women with high grade SILs. Simultaneous infection by HPV 6 and 11 was observed in 4 (9%) patients with low grade SILs and in 1 (4%) patient with a high grade SILs.

The relative risk calculated for HPV 16 was 3.4 and 3.8 in low and high grade SILs respectively. The relative risk for HPV 18 was 6.7 and 12.3 in low and high grade lesions respectively. High risk HPVs (16, 18, and 45)

TABLE II. HPV Genotypes Detected Among Patients and Controls in Senegal

	Cytology					
	Patients			Controls		
HPV types	Low grade N = 42	High grade N = 23	Total N = 65	Negative N = 26	Not obtained N = 46	Total N = 72
Negative	3 (7%)	1 (4%)	4 (6%)	20 (77%)	35 (76%)	55 (76%)
Any type	39 (93%)	22 (96%)	61 (94%)	6 (23%)	11 (24%)	17 (24%)
HPV 6	12 (29%)	5 (22%)	17 (26%)[a]	0 (0%)	0 (0%)	0 (0%)
HPV 11	6 (14%)	4 (17%)	10 (15%)[a]	0 (0%)	0 (0%)	0 (0%)
HPV 16	17 (40%)	10 (43%)	27 (42%)[a]	5 (19%)	7 (15%)	12 (17%)
HPV 18	14 (33%)	11 (48%)	25 (39%)[a]	1 (4%)	4 (9%)	5 (7%)
HPV 31	1 (2%)	0 (0%)	1 (1.5%)	0 (0%)	0 (0%)	0 (0%)
HPV 33	0 (0%)	1 (4%)	1 (1.5%)	0 (0%)	0 (0%)	0 (0%)
HPV 35	0 (0%)	0 (0%)	0 (0%)	—	—	—
HPV 45	5 (12%)	2 (9%)	7 (10%)	—	—	—
HPV 52	2 (5%)	0 (0%)	2 (3%)	—	—	—
HPV 58	0 (0%)	0 (0%)	0 (0%)	—	—	—
HPV 68	0 (0%)	1 (4%)	1 (1.5%)	—	—	—
Unclassified HPVs	4 (10%)	0 (0%)	4 (6%)	0 (0%)	0 (0%)	0 (0%)

[a]Including multiple infection.
HPV 35, 45, 52, 58 and 68 were sought in samples which amplified only with consensus primers or in HPV negative samples.

TABLE III. Prevalence of HPV in Senegalese Pregnant Women (N = 72) According to Age

Age (yr)	N	HPV positive (%)	95% CI
13–20	15	1 (6%)	0.2–31.9
21–25	14	3 (21%)	4.7–50.8
26–30	26	10 (39%)	30.3–59.4
31–43	17	3 (18%)	3.8–43.4

were detected in 66% of low grade SILs and in 78% of high grade SILs.

HPV prevalence was investigated among the pregnant women in relation to age (Table III). It was noted that HPVs were detected in only 6% (1/15) of women aged 13 to 20 years and in 39% (10/26) of women aged 26–30 years. However, the variation in HPV prevalence with age is not statistically significant.

DISCUSSION

HPV 16 was found in 42% of Senegalese women with intraepithelial lesions. This is in agreement with similar studies carried out in South Africa and Tanzania where HPV 16 was detected in 46% and 38% of invasive cervical cancers respectively [Williamson et al., 1994, and Ter Meulen et al., 1992]. HPV 18 was detected in 39% of cases from Senegal. This is similar to the 32% observed both in Tanzania [Ter Meulen et al., 1992] and in Uganda [Schmauz et al., 1989]. These data confirm that HPV 18 is frequent in certain African countries compared to the rest of the world where HPV 18 is detected in only 14% of cervical cancers [Bosch et al., 1995]. However, it must be noted that HPV 18 was detected only in 1.5% of cervical cancers in South Africa [Williamson et al., 1994]. HPV 45 was found more frequently in Senegal (10%) than in Tanzania (6%) and in South Africa (1.5%) [Ter Meulen et al., 1994 and Williamson et al., 1994]. The prevalence of HPV DNA in Senegalese women with normal cytology (23%) is higher than that observed in many countries but is similar to the 26% observed in the Oregon study [Hildesheim et al., 1994]. Moreover, a higher prevalence (59%) was reported for women from Tanzania [Ter Meulen et al., 1992].

In Senegal, however, the percentage (23%) of women with normal cytology infected with high risk HPV 16 and 18 is higher than that observed (6%) in Oregon and Uganda [Hildesheim et al., 1994; Schmauz et al., 1989] and than the 16% reported in Tanzania [Ter Meulen et al., 1992]. It is not clear whether the presence of HPV DNA in women with normal cervical cytology indicates very recent infection or whether it can predict future development of genital warts or dysplasia or both. The high prevalence observed among the Senegalese women with normal cytology could be explained by the fact that this group is composed of pregnant women and an increased prevalence of HPV infections has been reported in pregnant women compared to non-pregnant women [Czegledy et al., 1989]. However, other studies have not confirmed such an observation [Kemp et al., 1992]. This high frequency of HPV infections could also be attributed to simultaneous HIV infection in a high proportion of this population, since HPV 16 and HPV 18 have been reported to be 2.2 times more frequently detected in HIV positive asymptomatic women [Ter Meulen et al., 1992]. However, HIV 1 and HIV 2 have been detected in only 0.3% and 0.5% respectively of pregnant Senegalese women [Abbott et al., 1994], and this frequency could not explain the high HPV frequency observed in our study.

Multiple infections by several HPV types have been observed frequently in Senegal (28% of cases). The presence of more than one HPV type in a lesion has been previously described. Double infection with HPV 16 and HPV 18 have been reported in 5%–62% of cancers [Kristiansen et al., 1994; Rogo et al., 1991] and in 40% of cytologically normal women [Hildesheim et al., 1994]. In the present study, multiple infections were found only among patients but not in controls. Multiple HPV

infections could be attributed to multiple sexual contacts and a synergistic action of HPVs could have contributed to the development of cancers.

In conclusion, HPV infections are frequent in Senegal since HPV 16 and 18 were detected in 24% of pregnant women. Our data also confirmed that HPV 18 and 45 are detected frequently in Africa. HPV 18 is associated with cancers with poorer prognosis [Barnes et al., 1988]. In agreement with this finding, HPV 18 has been found to immortalise keratinocytes more efficiently than HPV 16 [Barbosa and Schlegel, 1989]. HPV 16 is the most common type found in cervical cancer but HPV 18 is associated with more aggressive neoplastic properties. The observation of a large number of patients in certain countries like Senegal with high risk HPVs, including a high proportion of HPV 18 infection, suggests that their populations are at an increased risk of developing more aggressive forms of cervical cancers.

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